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RELATIVE IMPORTANCE OF ALTERNATIVE PATHWAYS OF PURINE NUCLEOTIDE BIOSYNTHESIS IN EHRLICH ASCITES TUMOR CELLS *IN VIVO*

by

© CAMILLA MAY SMITH

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Relative importance of alternative pathways of purine nucleotide biosynthesis in Ehrlich ascites tumor cells *in vivo*", submitted by Camilla May Smith in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Several alternative pathways of purine nucleotide synthesis coexist in cells -- purine biosynthesis *de novo*, the purine phosphoribosyltransferases and adenosine kinase. However, the relative importance of each pathway for maintaining purine nucleotide concentrations in cells is not known. In this study, specific inhibitors were used to block these synthetic routes in Ehrlich ascites tumor cells *in vivo*, singly and in combination. The effect of inhibiting each pathway was evaluated by measuring intracellular purine nucleotide concentrations by high speed liquid chromatography. Purine biosynthesis *de novo* was inhibited by azaserine, mycophenolic acid inhibited inosinate dehydrogenase, hadacidin was used to inhibit adenylosuccinate synthetase and phosphoribosylpyrophosphate synthetase was inhibited by xylosyl adenine.

The results of this study indicate that adenosine triphosphate and guanosine triphosphate concentrations of Ehrlich ascites tumor cells are maintained primarily by purine biosynthesis *de novo* although other pathways do make significant contributions. The relative contributions of the synthetic pathways for maintaining normal guanosine triphosphate concentrations were 55% for purine biosynthesis *de novo*, 26% for hypoxanthine phosphoribosyltransferase and 19% for adenylate deaminase; those for maintaining normal adenosine triphosphate concentrations were 85% for purine biosynthesis *de novo*, 10% for hypoxanthine phosphoribosyltransferase and 5% for adenine phosphoribosyl transferase.

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LIST OF ABBREVIATIONS

AMP, ADP, ATP	5'-mono-, di- and triphosphates of adenosine
GMP, GDP, GTP	5'-mono-, di- and triphosphates of guanosine
IMP	inosine-5'-monophosphate
XMP	xanthosine-5'-monophosphate
NAD	nicotinamide adenine dinucleotide
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
m, μ, n	milli, micro, nano (10^{-3}, 10^{-6}, 10^{-9})
i.p.	intraperitoneal
I.D.	internal diameter
PPO	2,5-Diphenyloxazole
POPOP	p-bis-[2-(5-phenyloxazolyl)]benzene
M	moles per liter
N	gram equivalents per liter
PRPP	5-phosphoribosyl-1-pyrophosphate
APRT	adenine phosphoribosyltransferase
GPRT	guanine phosphoribosyltransferase
HPRT	hypoxanthine phosphoribosyltransferase
Ci	curie
cpm	counts per minute
PCA	perchloric acid
FGAR	formylglycineamide ribonucleotide (phosphoribosyl-formylglycineamide)
K_i	dissociation constant of the enzyme-inhibitor complex
AIR	aminoimidazole ribonucleotide (phosphoribosyl-aminoimidazole)
K_m	Michaelis constant
l	liter
g, gm	gram
m	meter

CHAPTER 1

INTRODUCTION

Several alternative pathways of purine nucleotide biosynthesis coexist in most animal cells (Fig. 1). These are purine biosynthesis *de novo*, the purine phosphoribosyltransferases which convert hypoxanthine, adenine and guanine to inosinate, adenylate and guanylate, respectively, and adenosine kinase which converts adenosine to adenylate. For only a few cells or tissues is the relative importance of these alternative pathways of purine nucleotide synthesis *in vivo* known. Studies of the regulation and biological significance of the purine biosynthetic enzymes have been reviewed (1, 2, 3).

Mammals do not require purines in their diet either for growth or maintenance (4, pp. 15-16), and purine biosynthesis *de novo* therefore can supply all the purine nucleotides required by the whole animal. However, this does not mean that this pathway operates in every cell or tissue. Mature human, rabbit and mouse erythrocytes lack one or more of the enzymes of purine biosynthesis *de novo*, although these enzymes are present in rabbit reticulocytes (4, pp. 204-205). In addition, human erythrocytes lack the ability to convert inosinate to AMP, presumably due to deficiency of adenylosuccinate synthetase (5, 6). Conflicting data exist regarding the ability of peripheral leukocytes to synthesize purine ribonucleotides *de novo* (yes: 7, 8; no: 9, 10), although it is clear that cultured lymphoblasts (11, 12) and phytohemagglutinin-stimulated leukocytes (13) can do so. On the basis of indirect evidence (14), it is generally believed that mam-

1. Phosphoribosyl pyrophosphate synthetase (EC 2.7.6.1) (PRPP synthetase)
2. Purine biosynthesis *de novo*
3. Hypoxanthine (guanine) phosphoribosyltransferase (EC 2.4.2.8) (HGPRT)
4. Adenine phosphoribosyltransferase (EC 2.4.2.7) (APRT)
5. Adenosine kinase (EC 2.7.1.20)
6. Adenylate deaminase (EC 3.5.4.5)
7. Guanylate reductase (EC 1.6.6.8)
8. Inosinate dehydrogenase (EC 1.2.1.14)
9. Guanylate synthetase (EC 6.3.5.2)
10. Adenylosuccinate synthetase (EC 6.3.4.4)
11. Adenylosuccinate lyase (EC 4.3.2.2)

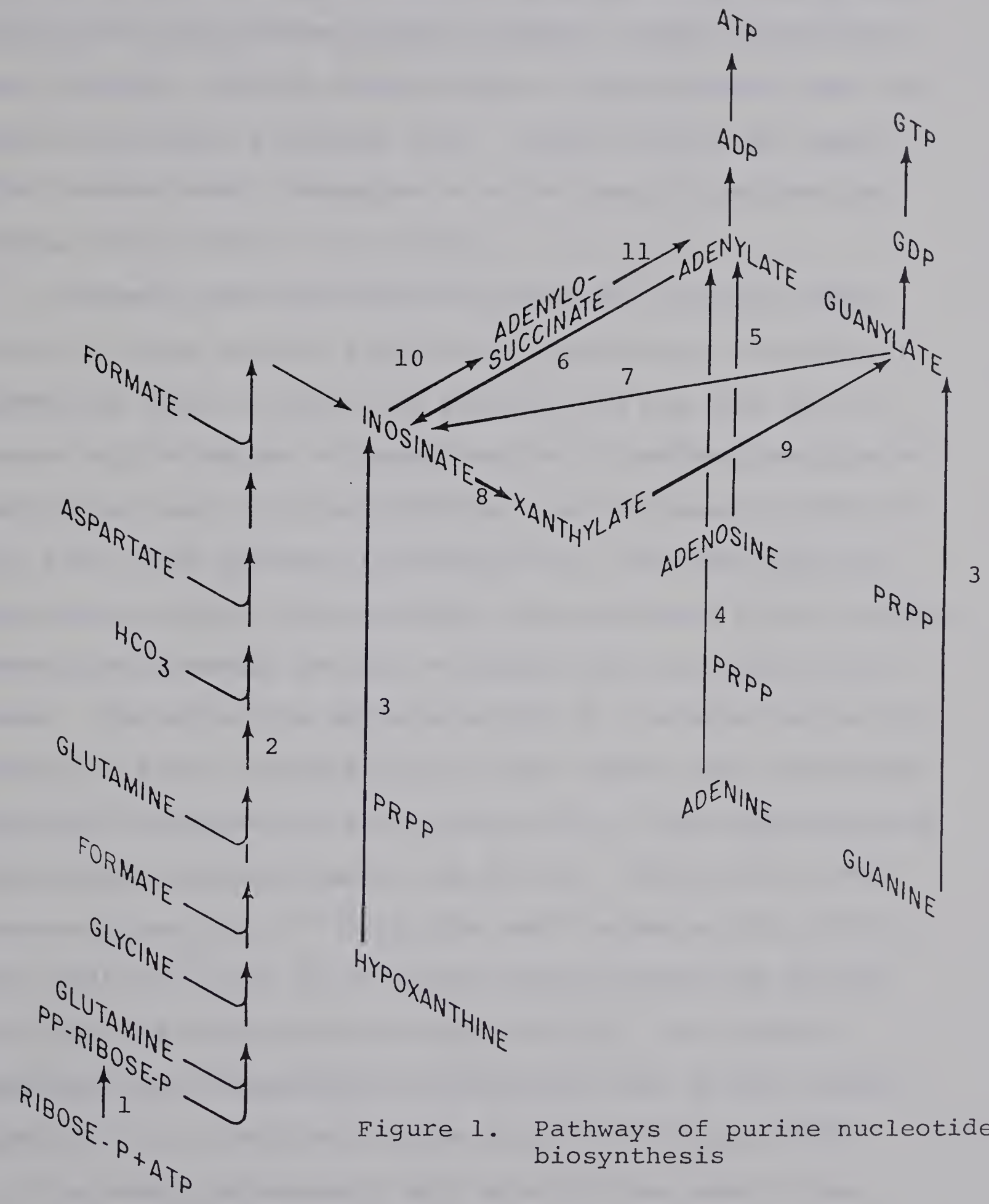


Figure 1. Pathways of purine nucleotide biosynthesis

malian bone marrow either cannot synthesize purine ribonucleotides *de novo* at all, or cannot do so at rates sufficiently rapidly for bone marrow growth; however, other explanations are possible. Purine biosynthesis *de novo* probably does not operate in blood platelets (15). There is also one report that hamster small intestine *in vitro* does not synthesize purine nucleotides *de novo* (16).

Attempts have been made to assess the relative importance of these several alternative biosynthetic reactions in mammalian cells *in vivo*. In general this has been done by comparing the extent of incorporation of radioactive glycine and purine bases into nucleotides. As discussed by Marko *et al.* (17) these studies are difficult to interpret, mainly because the normal intracellular concentrations of the purine bases are extremely low and at present are not accurately known. Therefore the administration of a labeled purine is likely to alter the pool size of that purine, and nucleotide synthesis consequently is measured only at the experimentally determined concentration of the purine. Thus, after simultaneous injection of ^{14}C-glycine and ^{3}H-adenine into chicks, the ratio of ^{14}C to ^{3}H in nucleic acid adenine and guanine of liver and intestine was measured (18). The results obtained were interpreted as indicating that purine biosynthesis *de novo* contributed more than did APRT plus HGPRT. In this study the measured pool size of free adenine was quite high, and the glycine pool size was only estimated; therefore, accurate intracellular specific activity measure-

ments were not carried out. A similar study was done for rat brain (19). ^{14}C-Formate or ^{14}C-glycine plus ^{3}H-adenine were injected intracerebrally and the ratio of ^{3}H to ^{14}C in the acid-soluble fraction of the cerebral hemispheres was determined. On the basis of this ratio, it was concluded that purine biosynthesis *de novo* has a greater functional significance than the other synthetic pathways. The previously mentioned criticisms also apply to this study.

Statement of thesis problem

A new approach to determining the relative contributions of the alternative pathways of purine nucleotide biosynthesis was undertaken in this study. The question asked was "What is the relative importance of each of the alternative pathways of purine nucleotide synthesis for maintaining normal concentrations of ATP and GTP in cells?" This problem was approached by administering drugs which specifically inhibit individual pathways of purine nucleotide biosynthesis and then measuring the intracellular concentrations of the purine nucleotides. The importance of a particular pathway was evaluated by the extent of reduction in ATP or GTP concentration after a defined period of continuous, constant inhibition. The assumptions made in following this approach will be discussed below.

The biological system used for this study was Ehrlich ascites tumor cells growing *in vivo*. The purine metabolism of these cells *in vitro* has been studied in considerable

detail (22, 23, 28, 58, 70, 73) and the administration of drugs and radioactive precursors and the technical manipulation of these cells are relatively simple.

CHAPTER 2

MATERIALS AND METHODS

Chemicals

Adenine-8-^{14}C (*ca.* 50 mCi/mmole) and glycine-^{14}C (U) (*ca.* 100 mCi/mmole) were purchased from New England Nuclear Corporation. Hypoxanthine-8-^{14}C (*ca.* 50 mCi/mmole) and guanine-8-^{14}C (*ca.* 50 mCi/mole) were from Schwarz BioResearch. Adenosine-8-^{14}C (*ca.* 50 mCi/mole) was purchased from New England Nuclear Corporation and from Schwarz BioResearch. The actual specific activity varied from lot to lot.

Non-radioactive purines, nucleosides and nucleotides were obtained from Sigma Chemical Co.

The following compounds were supplied by the Cancer Chemotherapy National Service Center (now called the Drug Evaluation Branch, Drug Research and Development), National Cancer Institute, Bethesda, Md.: azaserine (O-diazoacetyl-L-serine), hadacidin (N-formylhydroxyaminoacetic acid), 4-amino-5-iodo-7-β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine, N-6-phenyladenosine, 9-β-D-xylofuranosyladenine, and 2'-deoxycoformycin (NSC 218321). Mycophenolic acid was a gift from Dr. T. J. Franklin, Imperial Chemical Industries Ltd., Macclesfield, Cheshire, England. O-6-Benzylinosine was synthesized by Dr. Shambu Naik using a procedure developed by Dr. B. Paul (20). 9-Erythro(2-hydroxy-3-nonyl) adenine hydrochloride was obtained from Dr. H. J. Schaeffer, Wellcome Research Laboratories, Research Triangle Park, N. C.

Fischer's medium (21) for leukemic cells of mice (dry powder with L-glutamine) was purchased from Grand Island Biological Co. Fischer's "high phosphate" medium (hereafter

referred to simply as Fischer's medium) was made by the omission of bicarbonate and the addition of sodium phosphate to a final concentration of 25 mM. Polyethyleneimine cellulose thin layer chromatography sheets on Mylar film were purchased from Brinkman Instruments Limited, and cellulose thin layer chromatography sheets from Eastman Kodak Company. Polyethyleneimine cellulose powder was obtained from Sigma Chemical Company.

Preparation of Cell Extracts

Ehrlich ascites tumor cells were grown intraperitoneally in female ICR Swiss mice, supplied by the University of Alberta Laboratory Animal Breeding Service. Mice weighing 25 to 30 grams were implanted with approximately 5×10^7 cells (0.1 ml packed cells). After three days about 0.5 ml of packed cells was obtained.

To prepare cell extracts, the mice were killed by cervical dislocation and the cells were collected using cold Fischer's medium and transferred to 12-ml centrifuge tubes. The cells were sedimented by centrifugation for two minutes in a clinical centrifuge at 4° C. The ascitic fluid and collecting medium were discarded and the cells were extracted with 2.3 ml of cold 0.4 M perchloric acid. After 10 minutes, the extracts were centrifuged for 2 minutes and the pellets were re-extracted with 2.0 ml of cold 0.4 M perchloric acid. After 10 minutes, the extracts were centrifuged for 3.0 minutes and the first and second extracts were combined. To aid in

neutralization, the indicators phenolphthalein and bromcresol purple were added; at neutral pH these give a blue color. The perchloric acid extracts were neutralized to pH 6 with 4 M KOH and the extracts were centrifuged to remove the $KClO_4$ precipitate. 150 µl of 20 mM ascorbic acid was added to each extract (22). The extracts were then frozen to achieve maximum precipitation of $KClO_4$, and centrifuged again before analysis.

The above procedure was used when the analysis of purine nucleoside triphosphates was required. In experiments where total nucleotides were analyzed, several modifications of this procedure were used. The cells were collected at room temperature using Krebs-Ringer phosphate medium (23) and no ascorbic acid was added to the extracts.

Quantitation of Ehrlich ascites tumor cell numbers

The number of Ehrlich ascites tumor cells obtained from each mouse was not constant; therefore, some estimation of the cell number was required. The weight of perchloric acid-insoluble material obtained after preparation of the cell extract was used as a measure of the cell number.

To quantitate the relationship between cell number and the weight of acid-insoluble material, Ehrlich ascites tumor cell suspensions of varying cell density were prepared. Cell counts were determined using a Coulter counter. Each cell suspension was diluted in duplicate and two cell counts were determined for each diluted suspension.

The cells of each suspension were sedimented by centrifugation in pre-weighed tubes and extracted twice with perchloric acid as previously described. The tubes were then inverted and allowed to drain at 4^{o} C onto a paper towel. After 24 hours the weight of acid-insoluble material was determined.

Figure 2 shows a graph of the weight of acid-insoluble material *vs*. Ehrlich ascites tumor cell numbers. The slope of the line give the relationship 5.4×10^{8} cells per gram of perchloric acid-insoluble material.

For routine use, tumor cells were collected as described above using pre-weighed centrifuge tubes. After 24 hours, the tubes were re-weighed to determine the weights of perchloric acid-insoluble material.

Isolation of Radioactive Nucleotides

The neutralized cell extracts were desalted by chromatography on polyethyleneimine-cellulose columns. The procedure used was considerably modified from one described by Dr. Janet Oliver (personal communication to Dr. A. R. P. Paterson). A small amount of glass wool was placed in the bottom of each 0.9 cm I.D. glass column. A suspension of polyethyleneimine-cellulose in water was added until the column height was 5 cm. The column was washed with 4 ml of 1 M triethylammonium bicarbonate, pH 7.5, and then with 10 or more ml of water. The extract (about 5 ml) was mixed with 1 ml of methanol, centrifuged, and applied to the prepared column. The columns

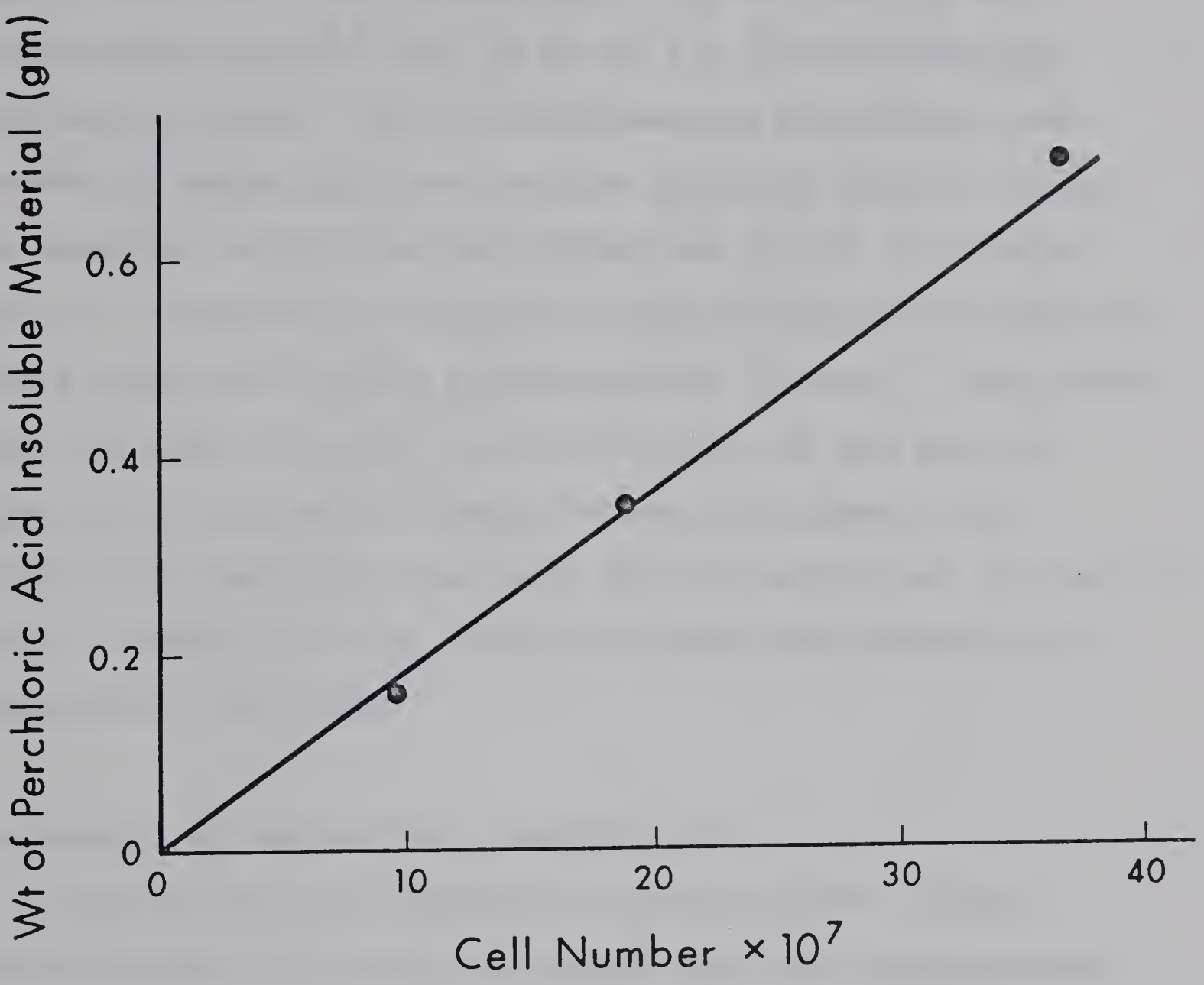


Figure 2. Relationship of weight of perchloric acid insoluble material to number of Ehrlich ascites tumor cells.

were washed with 4 ml of 80% methanol to remove salts and some of the bases and nucleosides. The nucleotides were eluted quantitatively with 10 ml of 1 M triethylammonium bicarbonate buffer. The triethylammonium bicarbonate was removed by evaporation over sodium hydroxide flasks *in vacuo*. The desalted nucleotides were dissolved in 0.2 ml of water and 10 µl portions were applied to polyethyleneimine-cellulose plates along with purine ribonucleotide carriers. The nucleotides GTP, ATP, GDP, ADP, GMP, AMP, IMP, XMP and NAD were separated by step-wise formate buffer development (23). Ultraviolet-absorbing areas were cut out and placed in counting vials. Toluene counting fluid was added and radioactivity measurements were made.

Measurement of nucleotide concentrations

Nucleotides were separated by high-pressure liquid chromatography on a Varian-Aerograph LCS-1000 chromatograph. The elution systems used were a linear gradient of potassium phosphate and potassium chloride at pH 4.5 (24) or a concave upward gradient of potassium phosphate and potassium chloride at pH 5.0. 10 µl of extract was used for each analysis. To quantitate the amount of individual nucleotides, peak areas were measured by planimetry and compared to standards of authentic nucleotides analyzed under the same conditions. A chromatogram of control cells is shown in Fig. 3.

ATP and GTP overlapped in some of the chromatograms; thus, the smaller GTP peak could not be accurately measured.

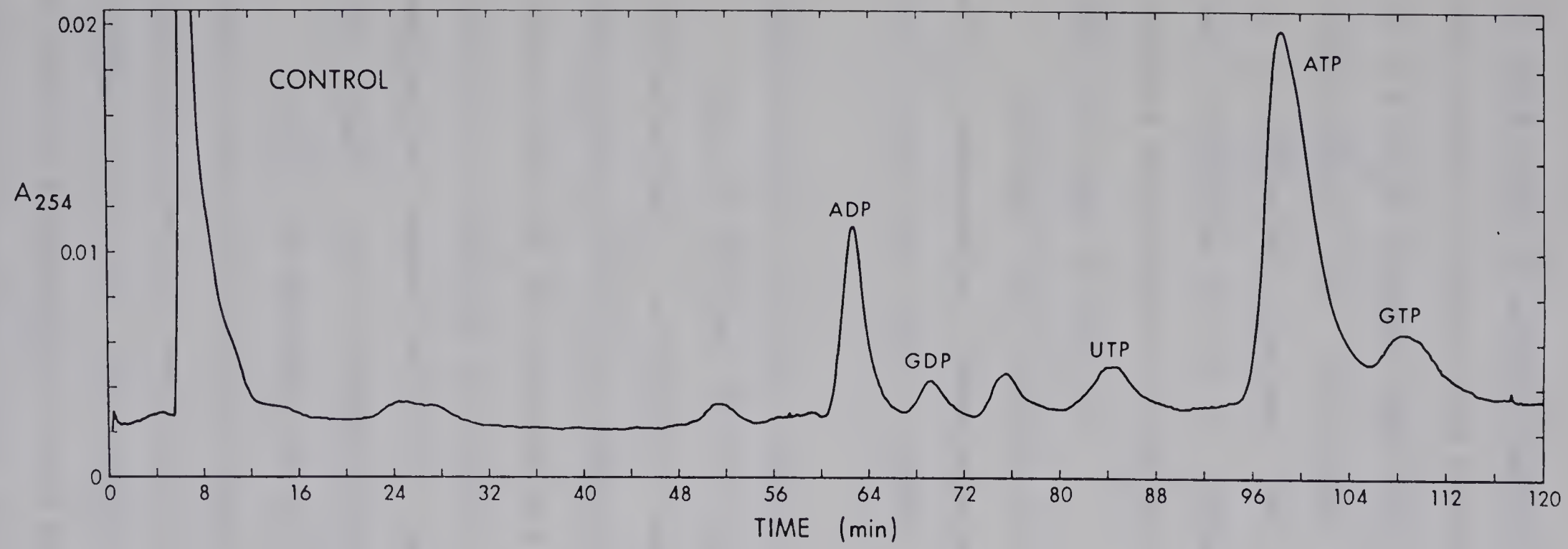


Figure 3. Nucleotides in Ehrlich ascites tumor cells.

The chromatograph was equipped with a 1 mm x 3 m Varian Aerograph column packed with PA-39 anion exchange resin or a 1 mm x 3 m Reeve Angel column packed with AS-Pellionex-SAX anion exchanger. The elution system used for the Varian Aerograph column was a linear gradient of 0.015 M KH_2PO_4, pH 4.5 and 0.25 M KH_2PO_4 - 2.2 M KCl, pH 4.5; the flow rate for the column was 12 ml per hour and for the high concentrate buffer was 6 ml per hour. The elution system used for the SAX column was a concave upward gradient of 0.015 M KH_2PO_4, pH 5.0 and 0.5 M KH_2PO_4- 1.0 M KCl, pH 4.5; the flow rate for the column was 15 ml per hour and for the high concentrate buffer was 10 ml per hour.

In this case, a second analyzer run was done on a portion of the extract treated with hexokinase to convert ATP to ADP. To 0.5 ml of extract was added 10 µl of 400 units/ml hexokinase (Sigma type C-130, from yeast), 5 µl of 20 mM $MgCl_2$ and 10 µl of 100 mg/ml glucose. The reaction mixture was incubated for 10 minutes at 37° C. Chromatography of the treated extract allowed measurement of the GTP peak without interference from ATP, as shown in Fig. 4.

Although the extracts were analyzed as soon as possible after preparation, there was no appreciable degradation of the nucleotides after 2 months of storage.

Determination of Total Guanine Concentrations

Xylosyl ATP and GTP did not separate on the Varian chromatograph. In order to measure guanine nucleotide concentrations, the xylosyladenine treated extracts were acid hydrolyzed and the guanine was isolated. The guanine concentration obtained for control cells by this method (512 nmoles/gm) agreed well with the GTP value obtained using high speed liquid chromatography (528 nmoles/gm).

2.0 Ml of extract and 100 µl of 11.7 M perchloric acid were placed in a boiling water bath for 60 minutes. The hydrolyzed extract was cooled and applied to a Dowex-50-H^+ column (1 x 3 cm). The column was washed with 10 ml of 0.1 N HCl and guanine was eluted with 10 ml of 6.0 N HCl. The guanine fraction was taken to dryness *in vacuo* and then dissolved in 0.2 ml of water. 150 µl was spotted on Whatman

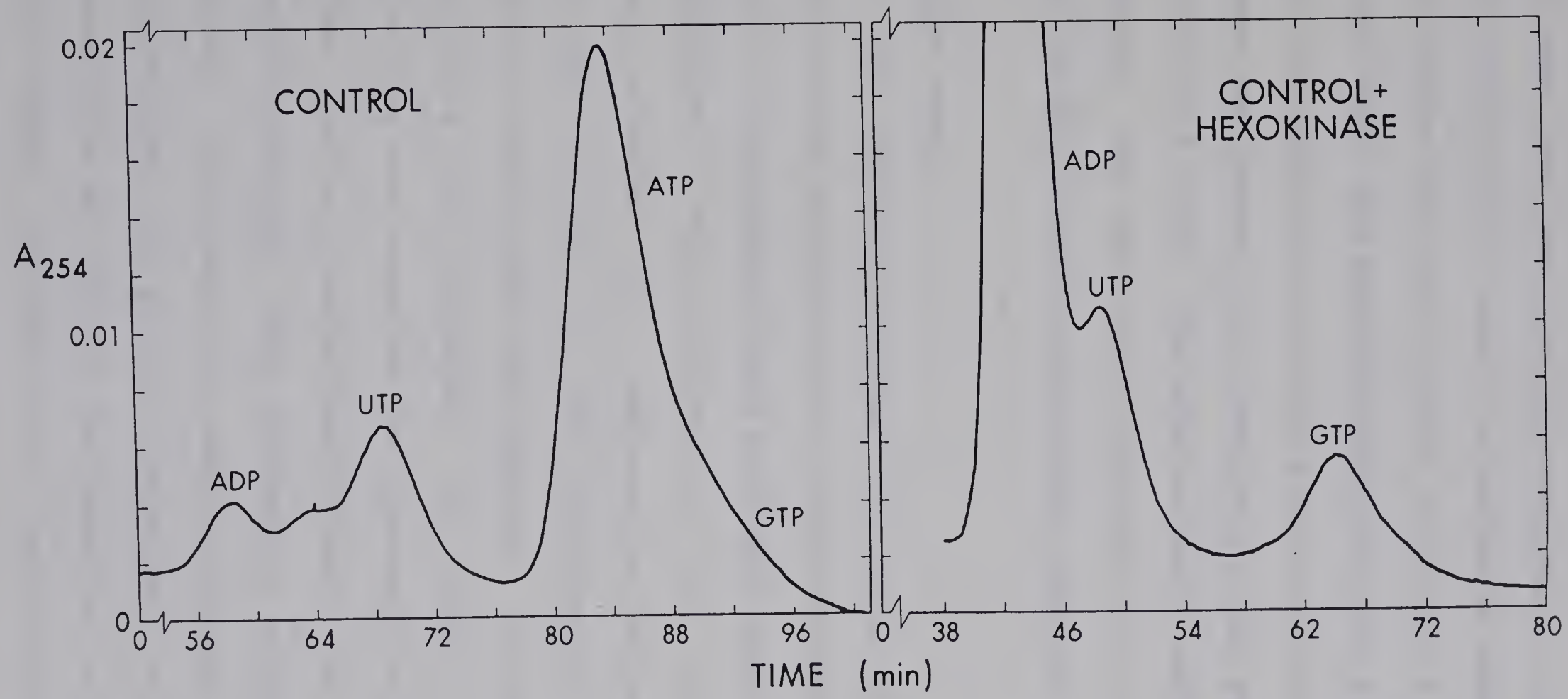


Figure 4. Nucleotides before and after hexokinase treatment of a control cell extract.

The high concentrate flow rate for the control was 6 ml per hour and for the control + hexokinase was 12 ml per hour.

#40 paper and developed (descending) in isopropanol-2.0 N HCl (65:35) for 24 hours, to separate guanine and adenine. The guanine spot was cut out, cut into strips and placed in a test tube. The paper was covered with 5 ml of 0.1 N HCl and left overnight to elute the guanine from the paper. The eluate was poured into a beaker and the paper was washed with 3 ml of 0.1 N HCl. The eluates were combined and taken to dryness. The guanine was dissolved in 1.0 ml of 0.1 N HCl and the absorbance at 248 nm was measured.

Enzyme Assays

The apparent activities of enzymes of purine nucleotide biosynthesis were measured in Ehrlich ascites tumor cells *in vivo*. The procedures used have been used in studies of purine metabolism in cells incubated *in vitro*, and are described in detail elsewhere (25). Radioactive purine bases and adenosine were injected i.p. into tumor-bearing mice, radioactivity in purine nucleotides was measured, and apparent enzyme activities were calculated. The enzymes or processes whose activities were measured are hypoxanthine phosphoribosyltransferase, guanine phosphoribosyltransferase, adenine phosphoribosyltransferase, PRPP synthetase, adenosine kinase, adenylosuccinate synthetase plus adenylosuccinate lyase, inosinate dehydrogenase, and purine biosynthesis *de novo*. Although hypoxanthine and guanine are converted to nucleotides by a single enzyme, the reaction of the two bases are considered as two separate processes.

Hypoxanthine phosphoribosyltransferase. Each of three tumor-bearing mice was injected i.p. with 3 μmoles of hypoxanthine-8-^{14}C (2 μCi/μmole). This amount of hypoxanthine was determined to be saturating for incorporation of radioactivity into purine nucleotides under these conditions (Table 1). After 30 minutes, the tumor cells were collected using Krebs-Ringer phosphate medium and cell extracts were prepared as previously described. 300 μl of the cell extract was spotted on Whatman 3MM paper along with nucleotide carriers. After development in n-butanol-acetic acid-water (25:15:10) for 24 hours (descending), the nucleotide area was cut out and its radioactivity was determined using toluene phosphor solution. The apparent activity of hypoxanthine phosphoribosyltransferase was equated to total radioactivity in purine nucleotides.

Guanine phosphoribosyltransferase. Each of three tumor-bearing mice was injected i.p. with 0.4 μmoles of guanine-8-^{14}C (50 μCi/μmole). This amount of guanine was determined to be saturating for incorporation of radioactivity into purine nucleotides under these conditions (Table 1). After 30 minutes, the cells were collected, a cell extract was prepared, and radioactivity in purine nucleotides was determined as described under hypoxanthine phosphoribosyltransferase. The apparent activity of guanine phosphoribosyltransferase was equated to radioactivity in purine nucleotides.

Table 1

Incorporation of radioactive purines and adenosine into purine nucleotides of Ehrlich ascites tumor cells *in vivo*.

Precursor	µmoles injected	Radioactivity in nucleotides (cpm per gm PCA insoluble material)
Hypoxanthine	0.4	2.1×10^5
(2 µCi/µmole)	3	5.8×10^5
Guanine	0.2	6.1×10^6
(50 µCi/µmole)	0.4	8.6×10^6
Adenine	0.3	3.0×10^5
(2 µCi/µmole)	3	9.4×10^5
Adenosine	1	2.4×10^5
(2 µCi/µmole)	2.5	2.0×10^5
	5.0	2.9×10^5

The labelling period was 30 minutes.

Adenine phosphoribosyltransferase. Each of three tumor-bearing mice was injected i.p. with 3 µmoles of adenine-8-^{14}C (2 µCi/µmole), which was determined to be near saturating for incorporation of radioactivity into purine nucleotides (Table 1). After 30 minutes, the cells were collected, a cell extract was prepared, and total radioactivity in purine nucleotides was determined. The apparent activity of adenine phosphoribosyltransferase was equated to total radioactivity in purine nucleotides.

Phosphoribosyl pyrophosphate synthetase. As PRPP is required for nucleotide synthesis from adenine, the apparent activity of PRPP synthetase was equated with that of adenine phosphoribosyltransferase. Evidence presented below indicates that PRPP synthesis is limiting for adenine phosphoribosyltransferase activity. Each of three tumor-bearing mice was injected i.p. with 3 µmoles of adenine-8-^{14}C (2 µCi/µmole), which was determined to be near saturating for incorporation of radioactivity into purine nucleotides (Table 1). After 30 minutes the cells were collected, a cell extract was prepared, and total radioactivity in purine nucleotides was determined. The apparent activity of PRPP synthetase was equated to total radioactivity in purine nucleotides.

Adenosine kinase. Measurement of adenosine kinase activity is complicated by the presence of adenosine deaminase in these cells. Therefore, 30 minutes prior to adenosine injection, an adenosine deaminase inhibitor 2'-deoxycoformycin

was administered i.p. at a dose of 1 mg/kg, which inhibits adenosine deaminase virtually completely for 24 hours (personal communication from Dr. G. A. LePage). Each of these mice was then injected i.p. with 1 µmole of adenosine-8-^{14}C (0.2 µCi/µmole) and sacrificed 30 minutes later. This amount of adenosine was determined to be near saturating for incorporation of radioactivity into nucleotides (Table 1). A cell extract was prepared and total radioactivity in purine nucleotides was determined. The apparent activity of adenosine kinase was equated to radioactivity in total purine nucleotides.

Adenylosuccinate synthetase plus adenylosuccinate lyase and inosinate dehydrogenase. Each of three tumor bearing mice was injected i.p. with 20 µl of hypoxanthine-8-^{14}C (50 µCi/µmole). After 30 minutes the cells were collected using cold Fischer's medium. The cell extract was prepared at 4^{o} C. After chromatography on a polyethyleneimine-cellulose column, the purine nucleotides were separated by thin layer chromatography on polyethyleneimine-cellulose plates. The apparent activity of adenylosuccinate synthetase plus adenylosuccinate lyase was equated to radioactivity in AMP, ADP, ATP and NAD. The apparent activity of inosinate dehydrogenase was equated to radioactivity in XMP, GMP, GDP, and GTP.

Purine biosynthesis de novo. Each of three tumor-bearing mice was injected i.p. with 50 µg of azaserine, which inhibits phosphoribosyl-formylglycineamidine synthetase, the fourth enzyme of purine synthesis *de novo* (26, 27). 30 Minutes later,

0.2 μmoles of ^{14}C-glycine (2 μCi/μmole) was injected. After another 30 minutes, the cells were collected using Krebs-Ringer solution and a cell extract was prepared. The accumulated phosphoribosylformylglycineamide (FGAR) was isolated by chromatography on Dowex-1-formate columns (28). Following application of the cell extract, the 1 x 3 cm column was washed with 30 ml of 0.05 M formic acid to remove ^{14}C-glycine and other metabolites. The radioactive FGAR was eluted from the column with 15 ml of 4 M formic acid. The FGAR fraction was evaporated to dryness *in vacuo*, dissolved in 1.0 ml of water, and 0.5 ml was mixed with 4.5 ml of Bray's counting solution and the radioactivity determined. The apparent activity of purine synthesis *de novo* was equated to radioactivity in the FGAR fraction.

Radioactivity Measurements

All radioactivity measurements were made using a Nuclear-Chicago Mark I liquid scintillation counter. Ultra-violet absorbing areas of thin layer sheets or paper were cut out, placed in a counting vial, and 18 ml of toluene phosphor solution (4 g PPO and 0.1 g POPOP per liter of toluene) was added. Radioactivity measurements were made for 4 minutes, with a counting efficiency of about 60%. Liquid samples were dissolved in 4.5 ml of Bray's scintillation liquid (29) and radioactivity measurements were made for 4 minutes. Actual measured cpm for controls ranged from 4,000 for inosinate dehydrogenase to 70,000 for GPRT.

CHAPTER 3

EFFICACY AND SPECIFICITY OF DRUGS USED TO INHIBIT PURINE NUCLEOTIDE BIOSYNTHESIS

Introduction

Evaluation of the relative importance of each of the purine biosynthetic routes for maintaining normal nucleotide concentrations in this study required inhibiting one or more of the pathways for a specified time by administration of drugs. Ideally, drugs used would inhibit nearly completely only one particular enzyme and the extent of inhibition would be constant over the period of the experiment.

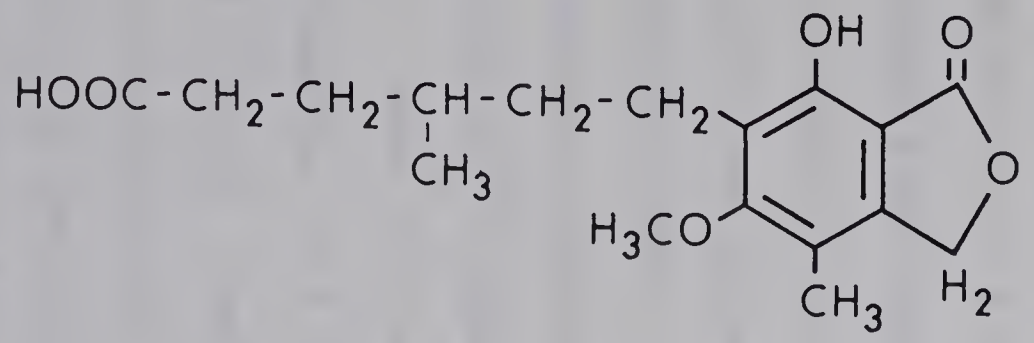
The time of the experiment was chosen as 4 hours on the basis of preliminary experiments with azaserine, a drug which inhibits purine biosynthesis *de novo* virtually completely for 12 hours after administration (30). Although nucleotide concentrations decreased for up to 11 hours after injection of azaserine, the decrease was linear only for the first 4 hours during which time adenine nucleotide concentrations dropped from 2860 nmoles/gm to 1300 nmoles/gm.

Other drugs used to inhibit reactions of purine nucleotide synthesis were mycophenolic acid, hadacidin, and xylosyl adenine. Drug treatment schedules (dose and number of treatments) required to produce a relatively constant inhibition during the 4 hour period were determined for each drug. The degree of inhibition obtained and the effect of each drug on other reactions of purine metabolism during the experimental period were also determined.

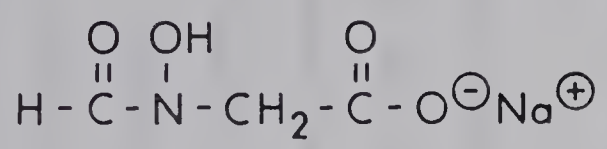
Literature Review

Xylosyl adenine. 9-β-D-Xylofuranosyladenine, a nucleoside analog (Fig. 5), is an inhibitor of PRPP synthetase. Following i.p. injection of tumor-bearing mice, xylosyl adenine was

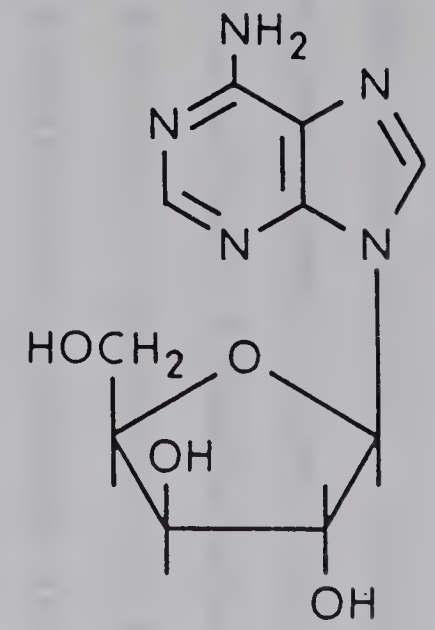
MYCOPHENOLIC ACID



HADACIDIN



XYLOSYLADENINE



AZASERINE

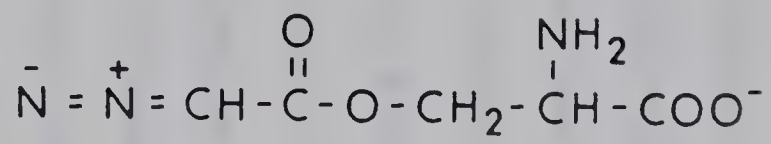


Figure 5. Chemical structures of drugs used to inhibit purine nucleotide synthesis.

shown to be rapidly distributed throughout the body, but little or no drug was found in the brain or bone marrow (31). When mice were administered 1 mg of xylosyl adenine, the highest concentration of drug was found in the tumor cells (15% of the dose after 15 min). In the cell, xylosyl adenine is readily phosphorylated, primarily to the triphosphate, or is deaminated to xylosyl hypoxanthine, the major excretory product. 60 Percent of the drug was excreted within 3 hours.

Xylosyl adenine was effective in prolonging the survival time of mice bearing Ehrlich and TA3 ascites tumor cells (32). Four daily treatments of 25 mg/kg of xylosyl adenine started 48 hours after implantation of Ehrlich cells increased survival time from 15 to 23.5 days. Xylosyl adenine treatment for 4 days at 25 mg/kg also suppressed the growth of TA3 ascites tumor cells. However, a daily dose of 50 mg/kg for 5 days resulted in a 20% loss in body weight, but had no effect on the blood leukocyte level.

Xylosyl adenine inhibited the incorporation of adenine-8-^{14}C and glycine-2-^{14}C into acid-soluble and nucleic acid fractions (32). When TA3 ascites cells were incubated *in vitro* in the presence of 2.0 mM xylosyl adenine, and labeled with adenine-8-^{14}C for 1 hour, the total radioactivity of acid-soluble nucleotides was 25% of untreated controls, the specific activity of RNA adenine was reduced 92% and that of DNA adenine was reduced 87%. When tumor cells were treated with azaserine to inhibit phosphoribosyl formylglycineamidine synthetase, 1 mM xylosyl adenine inhibited FGAR accumulation

by 70%. The presence of xylosyl adenine prevented the formation of PRPP from glucose in Ehrlich ascites tumor cells. Xylosyl adenine did not affect the incorporation of glycine-2-^{14}C into protein.

Using a cell-free extract of TA3 ascites tumor cells, Ellis and LePage (33) have shown that xylosyl adenine and xylosyl ATP did not affect the conversion of PRPP and labeled adenine to nucleotides. But the synthesis of labeled nucleotides from ribose-5-P, ATP and adenine-8-^{14}C was inhibited 85% by 0.8 mM xylosyl ATP. Unphosphorylated xylosyl adenine did not inhibit nucleotide synthesis from ribose-5-P, ATP and ^{14}C-adenine.

Xylosyl adenine triphosphate inhibits PRPP production, causing the observed inhibition of adenine and glycine incorporation into the acid-soluble nucleotide pool and into RNA and DNA. Since the inhibitions of incorporation of adenine into RNA and DNA were greater than the inhibition of incorporation into acid-soluble nucleotides, xylosyl adenine may also inhibit RNA and DNA synthesis.

Mycophenolic acid. Mycophenolic acid (Fig. 5) inhibits the conversion of inosinate to guanylate. After i.p. or oral administration of mycophenolic acid, the only metabolite of the drug was the glucuronide, in which the phenolic hydroxyl group is substituted by glucuronic acid (34). Glucuronides in general are considered to be biologically inert; mycophenolic acid glucuronide was 2000 times less active against inosinate dehydrogenase *in vitro* than free mycophenolic acid

(35). In mouse plasma, about 50% of the drug is conjugated with glucuronic acid (36). Glucuronides cannot cross cell membranes, but must be hydrolyzed by β-glucuronidase. There is some correlation between β-glucuronidase activity of tumors and inhibition of their growth (34). Mycophenolic acid is secreted into the bile and excreted in the urine and feces (36). In the urine 90-100% of the dose is recovered as mycophenolic acid glucuronide.

Mycophenolic acid at a concentration of 0.1 µg/ml stopped the incorporation of ^{14}C-thymidine into DNA of L-cells (37). The inhibition of DNA synthesis by mycophenolic acid was reversed by 1 µg/ml guanine. Mycophenolic acid at a concentration of 4.4 µM depressed the growth of L-cells in purine-free Eagle's medium to 22% of control (36). Xanthosine or inosine added to the medium did not reverse the effect of mycophenolic acid while guanosine at 10 times the concentration of mycophenolic acid resulted in 84% of control growth. Mycophenolic acid at 50 µg/ml caused about 90% inhibition of the incorporation of ^{14}C-hypoxanthine into acid-soluble and acid-insoluble guanine of Yoshida ascites cells and Landschütz ascites cells, while incorporation into acid-soluble and acid-insoluble adenine was not reduced (37).

These results imply that mycophenolic acid interferes with the conversion of inosinate to guanylate. Partially purified inosinate dehydrogenase and GMP synthetase from Landschütz ascites cells were inhibited by mycophenolic acid with K_i's of 3×10^{-8} M and 8×10^{-8} M respectively (36).

Inosinate dehydrogenase from different sources has a K_m for inosinate of 2×10^{-5} M (38, p. 145). Mycophenolic acid at a concentration of 0.1 μM resulted in an apparent inhibition of inosinate dehydrogenase of 72% in Ehrlich ascites tumor cells incubated *in vitro* in the presence of ^{14}C-hypoxanthine but GMP synthetase and other reactions of purine nucleotide interconversion were not significantly affected (39).

FGAR accumulation in azaserine-treated Yoshida ascites cells, Landschütz ascites cells, and L-cells was reduced 19-39% by 10 μg/ml mycophenolic acid (37). This effect is likely to be minor compared to its effect on inosinate dehydrogenase (and GMP synthetase). An observed partial correlation between high HGPRT activity and resistance to mycophenolic acid may be due to GMP synthesis via GPRT (36).

Hadacidin. N-Formyl hydroxyaminoacetic acid, an aspartic acid analog (Fig. 5), which was given the trivial name hadacidin because of its inhibitory action against human adenocarcinoma-1 (HAd-1) growing in the embryonated egg, is an inhibitor of adenylosuccinate synthetase (40). Hadacidin at a concentration of 1.3 mM inhibited the incorporation of ^{14}C-glycine into acid-insoluble adenine of Ehrlich ascites tumor cells *in vitro* by 95%, but did not affect ^{14}C-glycine incorporation into acid insoluble guanine (41). At a concentration of 0.87 mM, hadacidin inhibited the incorporation of ^{14}C-hypoxanthine into acid-insoluble adenine by 92% but did not affect the incorporation into acid-insoluble guanine in Ehrlich ascites tumor cells *in vitro*. Hadacidin did not inhibit the incorpora-

tion of ^{14}C-adenine or ^{14}C-guanine into acid-insoluble material. Using a cell-free extract of Ehrlich ascites tumor cells, the inhibition of incorporation of ^{14}C-hypoxanthine into acid-soluble AMP was partially reversed in the presence of excess aspartate. Hadacidin (310 mg) injected i.p. into rats reduced the incorporation of ^{14}C-glycine into RNA adenine and DNA adenine of intestinal mucosa, spleen and thymus. Shull (42) found that while hadacidin (106 mg) blocked the effect of inosine in preventing ATP reduction in rat liver during ethionine treatment, hadacidin treatment alone did not affect hepatic ATP levels. Shigeura (43) prepared adenylosuccinate synthetase from *Escherichia coli*, strain B, and measured adenylosuccinate formation. With equimolar amounts of hadacidin and L-aspartate there was no significant formation of product. Incubation with either aspartate or hadacidin prior to assay did not alter the rate of adenylosuccinate formation, indicating that there was no irreversible binding. As the ratio of aspartate to hadacidin was increased, the inhibition of adenylosuccinate synthetase decreased. Competitive inhibition kinetics were observed at three different concentrations of aspartate. At pH 8, adenylosuccinate synthetase has a K_m for aspartate of 1.5×10^{-4} M and a K_i for hadacidin of 4.2×10^{-6} M. These experiments indicated a lack of reaction between inosinate and hadacidin and a lack of effect on adenylosuccinate lyase activity.

Hadacidin at 4 to 6 mM inhibited the growth of KB cell cultures by 50% (44). In the presence of 8 mM hadacidin,

pyruvate and oxalacetate promoted the growth of the cultures, perhaps through conversion to aspartate. Adenine at concentrations of .02 to 0.1 mM reversed the cytotoxicity of 6 mM hadacidin, while adenosine, hypoxanthine, and inosine were ineffective. The cytotoxicity of hadacidin was enhanced by phenethyl biguanide, a respiratory inhibitor, indicating that hadacidin may affect energy metabolism as a consequence of its effect on ATP synthesis.

Azaserine. Azaserine, a glutamine analog (Fig. 5), is an inhibitor of phosphoribosyl formylglycineamidine synthetase, the fourth enzyme of purine biosynthesis *de novo*. The effect of azaserine on purine metabolism has been recently reviewed by Henderson (4, pp. 223-232).

Azaserine inhibited the incorporation of glycine-2-^{14}C into purines of Ehrlich ascites tumor cells *in vitro* and *in vivo* (30). At a concentration of 0.008 µg/ml (sic; probably 1.0 µg/ml), azaserine inhibited the incorporation of ^{14}C-glycine into acid-soluble adenine of Ehrlich ascites tumor cells *in vitro* by 96% and that into nucleic acid adenine by 92%. One injection of 5 µg of azaserine inhibited ^{14}C-glycine incorporation into acid-soluble adenine of Ehrlich ascites tumor cells *in vivo* by 99% after 3 hours, 86% after 12 hours, and 71% after 24 hours; the incorporation into nucleic acid adenine was inhibited 100% after 3 hours while the incorporation into protein was not affected. At a dose of 4 mg/kg, azaserine inhibited the incorporation, in solid Ehrlich carcinomas *in vivo*, of ^{14}C-glycine into nucleic acid adenine

and nucleic acid guanine 80% and 92%, respectively (45). At a dose of 0.4 mg/kg, azaserine increased the incorporation of adenine-8-^{14}C into nucleic acid adenine of Ehrlich ascites tumor cells *in vivo* (46) while incorporation of ^{14}C-adenine into RNA and DNA of Sarcoma 180 ascites cells *in vivo* was reported to be unaffected by azaserine (47). At a dose of 0.2 mg/kg, azaserine resulted in a maximum reduction of 60% in the adenine nucleotide concentration of Sarcoma 180 ascites tumor cells. Injection of adenine restored the adenine nucleotide concentration to control values. The survival time of mice bearing Ehrlich ascites tumor cells was increased from 15 days to 18 days with 6 daily injections of 0.4 mg/kg azaserine started 24 hours after implantation and was increased to 21 days with 2 injections per day of 0.2 mg/kg for 6 days (30).

Azaserine is concentrated intracellularly in Ehrlich ascites tumor cells by an amino acid active transport system (48). After a single injection, azaserine was removed from the blood very rapidly in mice but more slowly in humans (45). Mouse liver contains an enzyme which rapidly degrades azaserine with a K_m of 2×10^{-3} M at pH 7.4 (49). The breakdown of azaserine by Ehrlich ascites tumor cells was less than 1% of that by liver.

Azaserine irreversibly titrates phosphoribosyl formylglycineamidine synthetase by an alkylation reaction with a particular sulfhydryl group of the enzyme (50), and in cells this results in accumulation of FGAR. When the pigeon liver

enzyme was preincubated with azaserine, the addition of glutamine and other substrates at a later time did not restore enzyme activity (26). When glutamine was incubated simultaneously with azaserine, the inactivation of the enzyme was delayed. A partially purified enzyme from Ehrlich ascites tumor cells had a K_m for glutamine of 1.0×10^{-4} M and a K_i for azaserine of 2.3×10^{-6} M (51). Azaserine had no significant effect on the conversion of AIR to inosinate by a pigeon liver extract (52).

A secondary consequence of the inhibition of purine biosynthesis *de novo* by azaserine was a 75% reduction in thymidine kinase activity in Sarcoma 180 ascites tumor cells (53). Thymidine kinase is stabilized by ATP, and the reduction in ATP caused by azaserine may have increased the degradation of the enzyme.

Inhibition of enzyme activities

PRPP synthetase. Inhibition of PRPP synthetase of Ehrlich ascites tumor cells *in vivo* by xylosyl adenine was assayed as described in the methods section. Mice were injected with ^{14}C-adenine and the radioactivity in nucleotides was determined. The xylosyl adenine was dissolved in 0.9% saline, which was made pH 5 with HCl to increase solubility. The concentration of the drug solution was 20 mM (5.34 mg/ml).

Two injections of xylosyl adenine of 5 μmoles each, given at zero and 30 minutes, resulted in an apparent inhibition of 94% when PRPP synthetase activity was assayed at

1 hour.* When enzyme activity was assayed at 4 hours, the apparent inhibition was 62%. The drug schedule finally used to inhibit PRPP synthetase for over a 4 hour period was 10 μmoles at zero time and 5 μmoles each at 1, 2 and 3 hours. This treatment gave an apparent inhibition of PRPP synthetase of 84% at 4 hours.

Table 2 shows the effect of xylosyl adenine treatment on reactions of purine metabolism. All of the PRPP requiring reactions are greatly inhibited. The other reactions are not significantly affected.

Inosinate dehydrogenase. The inhibition of inosinate dehydrogenase of Ehrlich ascites tumor cells *in vivo* by mycophenolic acid was assayed as described in the methods section. Mycophenolic acid was dissolved in 0.15 M $NaHCO_3$. The concentration of the drug solution was 1 mM (0.32 mg/ml). One injection of 0.4 μmoles of mycophenolic acid resulted in an apparent inhibition of inosinate dehydrogenase of 87% after 1 hour and an inhibition of 69% after 4 hours. The drug schedule finally used to inhibit inosinate dehydrogenase over a 4 hour period was 0.5 μmoles of mycophenolic acid at zero time and 0.5 μmoles at 2 hours. This treatment resulted in an apparent inhibition of inosinate dehydrogenase of 80% at 4 hours.

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* Enzyme activity was always assayed during the 30 minutes prior to the time indicated.

Table 2

Effect of Inhibitors on Reactions of Purine Nucleotide Biosynthesis

Drug	Enzyme or Process						
	Purine Biosynthesis *De Novo*	HPRT	APRT	GPRT	Adenosine Kinase	Adenylosuccinate Synthetase & Lyase	Inosinate Dehydrogenase
	% Inhibition						
Xylosyl adenine	98	92	84	86	11	-*	-*
Mycophenolic Acid	16	6	-38	39	-19	11	80
Hadacidin	-18	43	0	1	6	87	-88
Azaserine	99	-70	-60	-89	-19	9	-29

Drug schedules. Xylosyl adenine: 10 µmoles at zero time and 5 µmoles at 1, 2 and 3 hours. Mycophenolic Acid: 0.5 µmoles at zero time and at 2 hours. Hadacidin: 15 µmoles at 0, 1, 2 and 3 hours. Azaserine: 20 µg at zero time. Apparent enzyme activities at 4 hours were determined as described in Chapter 2. A negative value for % inhibition means an increase in apparent activity.

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* not determined

Table 2 shows the effect of mycophenolic acid treatment on reactions of purine metabolism. The apparent activity of GPRT was reduced 39% and that of APRT was increased 38%; the other reactions were not significantly affected.

Adenylosuccinate synthetase. The inhibition of adenylosuccinate synthetase of Ehrlich ascites tumor cells *in vivo* by hadacidin was assayed as described in the methods section. Hadacidin was dissolved in 0.15 M $NaHCO_3$. The concentration of the drug solution was 75 mM (10.6 mg/ml). One injection of 30 μmoles of hadacidin resulted in an apparent inhibition of adenylosuccinate synthetase activity of 92% at 1 hour and 46% at 4 hours. The drug schedule finally used to inhibit adenylosuccinate synthetase over a 4 hour period was 4 hourly injections of 15 μmoles each. This treatment resulted in an apparent inhibition of adenylosuccinate synthetase of 87% at 4 hours.

Table 2 shows the effect of hadacidin on reactions of purine metabolism. There was an apparent inhibition of HPRT of 43% and, as a consequence of the inhibition of adenylosuccinate synthetase, there was an apparent increase of 88% in inosinate dehydrogenase activity. The other reactions were not significantly affected.

Purine biosynthesis de novo. Purine biosynthesis *de novo* of Ehrlich ascites tumor cells *in vivo* was inhibited by a single injection of 20 μg of azaserine, a dose which inhibits the incorporation of ^{14}C-glycine into purines by 95%

for 12 hours (30). Azaserine was dissolved in distilled water at a concentration of 1 mg/ml.

Table 2 shows the effect of azaserine treatment on reactions of purine metabolism. As a consequence of the inhibition of purine biosynthesis *de novo* there was an apparent increase in the activities of the PRT reactions -- 70% increase for HPRT, 60% increase for APRT, and 90% increase for GPRT. Adenosine kinase, adenylosuccinate synthetase, and inosinate dehydrogenase activities were not affected by azaserine.

Other reactions. Specific inhibitors were not found for APRT, HGPRT, or adenosine kinase activities in Ehrlich ascites tumor cells *in vivo*.

1-Methyl adenine at a concentration of 1.0 mM was reported to inhibit partially-purified APRT from Ehrlich ascites tumor cells by 90% (54). However, at a concentration of 1 mM, 1-methyl adenine inhibited the incorporation of ^{14}C-adenine into nucleotides of Ehrlich ascites tumor cells incubated *in vitro* only 13%, and injection of 50 µmoles of 1-methyl adenine into tumor-bearing mice did not affect the incorporation of ^{14}C-adenine into nucleotides of Ehrlich ascites tumor cells *in vivo* at 1 hour.

6-Benzylinosine at a concentration of 1 mM inhibited the incorporation of ^{14}C-hypoxanthine into purine nucleotides of Ehrlich ascites tumor cells *in vitro* by 63% (personal communication from Dr. J. F. Henderson) and did not inhibit purine biosynthesis *de novo*. Four hourly injections of 20 µmoles each caused an apparent inhibition of HPRT of 76% in Ehrlich ascites

tumor cells *in vivo* but also resulted in an apparent inhibition of purine biosynthesis *de novo* of 98%. When the dose of 6-benzylinosine was reduced so that purine biosynthesis *de novo* was not inhibited, HPRT activity was also not inhibited.

Several potential inhibitors of adenosine kinase were tested. 4-Amino-5-iodo-7-β-D-ribofuranosyl-7H-pyrrolo[2,3-d]pyrimidine, at a concentration of 0.1 mM was reported to inhibit adenosine kinase activity 94% in a cell-free extract of Ehrlich ascites tumor cells (55). One injection of 0.06 μmoles of this drug caused an apparent inhibition of adenosine kinase of 74% at 4 hours in Ehrlich ascites tumor cells *in vivo*, but reduced the apparent activity of purine biosynthesis *de novo* by 64%. At a lower dose, there was no apparent inhibition of either adenosine kinase or purine biosynthesis *de novo*. Phenyladenosine has also been reported to be an inhibitor of adenosine kinase (56). One injection of phenyladenosine (37.5 mg/kg) resulted in an apparent inhibition of 32% for Ehrlich ascites tumor cells *in vivo* but also caused a 59% reduction in the apparent rate of purine biosynthesis *de novo*. 9-Erythro-(2-hydroxy-3-nonyl) adenine hydrochloride, a potent inhibitor of adenosine deaminase (57), causes some inhibition of adenosine kinase at high doses. Preliminary experiments using Ehrlich ascites tumor cells *in vitro* showed that a maximum inhibition of adenosine kinase activity of 59% was obtained using 20 μg/ml of the drug (personal communication from Mr. G. Zombor). Injection of 2 mg of the drug inhibited the apparent activity of adenosine kinase of Ehrlich ascites tumor cells *in vivo* only 37%.

CHAPTER 4

RELATIVE IMPORTANCE OF ALTERNATIVE PATHWAYS OF PURINE NUCLEOTIDE SYNTHESIS IN EHRLICH ASCITES TUMOR CELLS *IN VIVO*

Purine Nucleotide Concentrations

The conditions required to inhibit various of the pathways of purine nucleotide synthesis for a 4 hour period were described in Chapter 3. The importance of a particular pathway was then evaluated by measuring the extent of reduction in ATP or GTP concentrations after 4 hours of continuous, relatively constant inhibition; these data and calculations are described in this chapter.

Figs. 6-10 show the nucleotide chromatograms obtained for control cells and those treated with mycophenolic acid, hadacidin, xylosyl adenine or azaserine. (Fig. 6 is the same as Fig. 3, previously shown, and is repeated here for reference.)

Fig. 6 shows the nucleotide profile of untreated control Ehrlich ascites tumor cells. The large initial peak contains bases, nucleosides and NAD. The majority of the nucleotides are in the triphosphate form.

Fig. 7 is a chromatogram of an extract of Ehrlich ascites tumor cells treated *in vivo* with mycophenolic acid for 4 hours. The GTP peak is very much reduced.

Fig. 8 shows the nucleotide profile of Ehrlich ascites tumor cells treated *in vivo* with hadacidin for 4 hours. The ATP peak is diminished while the GTP peak is larger than for control cells.

Fig. 9 is a chromatogram of an extract of Ehrlich ascites tumor cells treated *in vivo* with xylosyl adenine for 4 hours. The ATP peak is reduced and there is at least twice as much

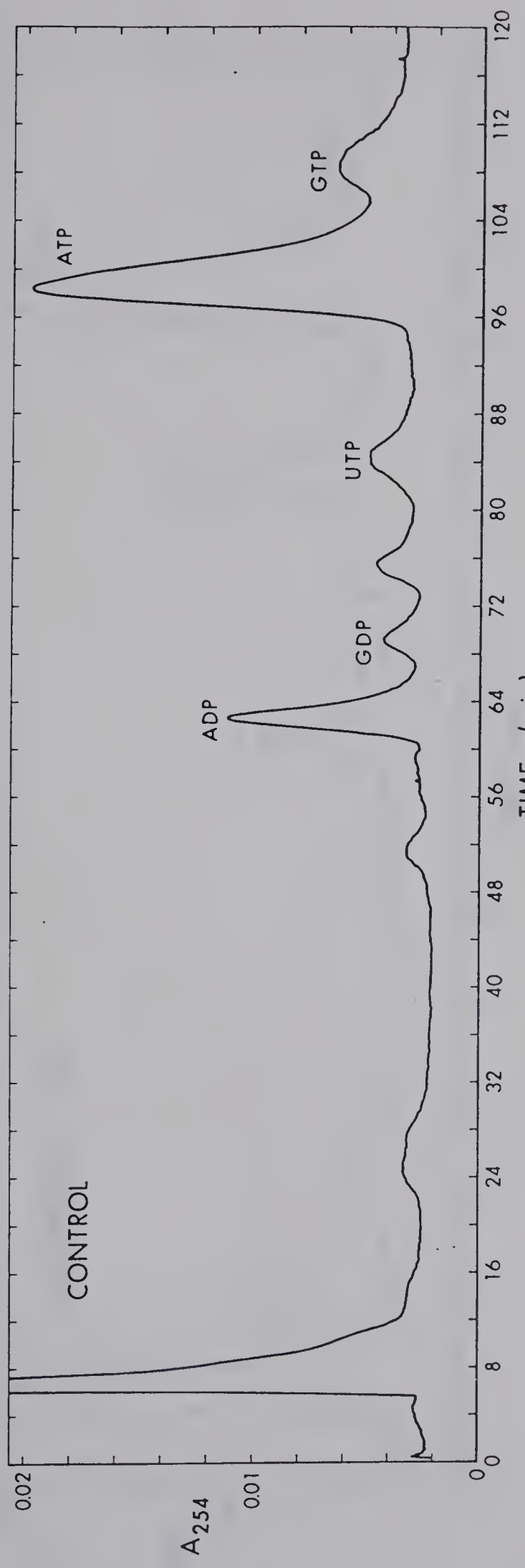

Figure 6. Nucleotides in untreated Ehrlich ascites tumor cells.

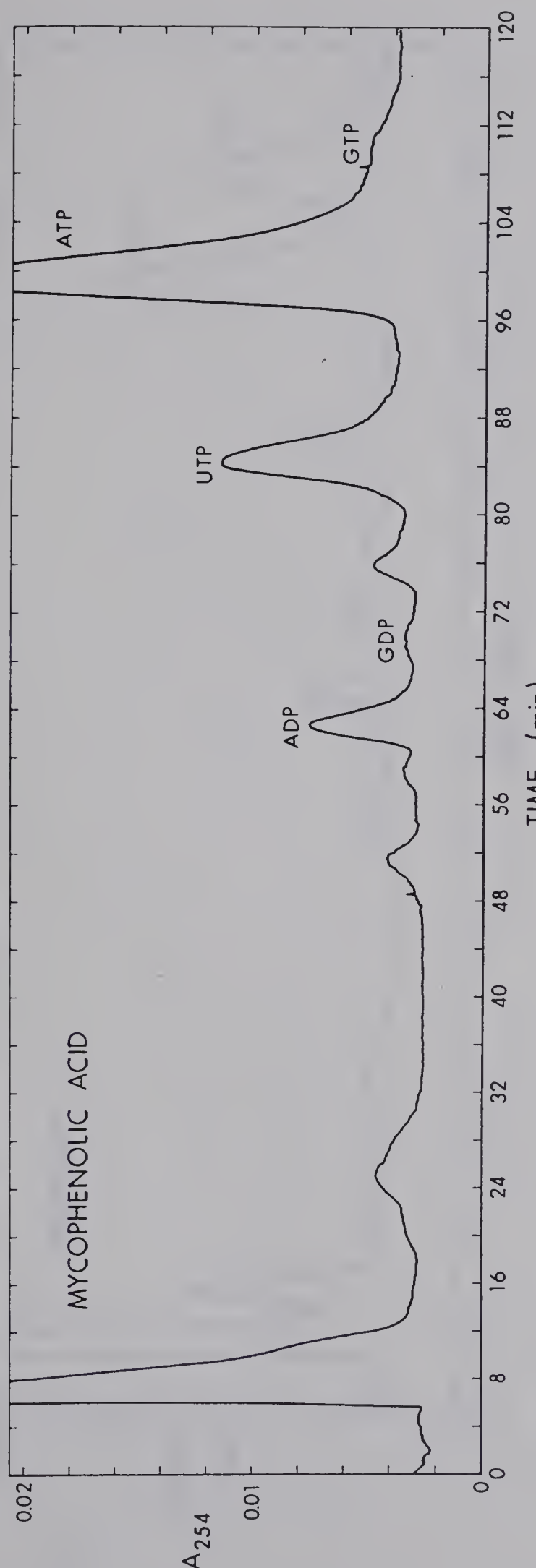

Figure 7. Chromatogram of cells treated with mycophenolic acid.

Mice were injected with 0.5 µmoles of mycophenolic acid at zero and 2 hours. Cells were collected at 4 hours.

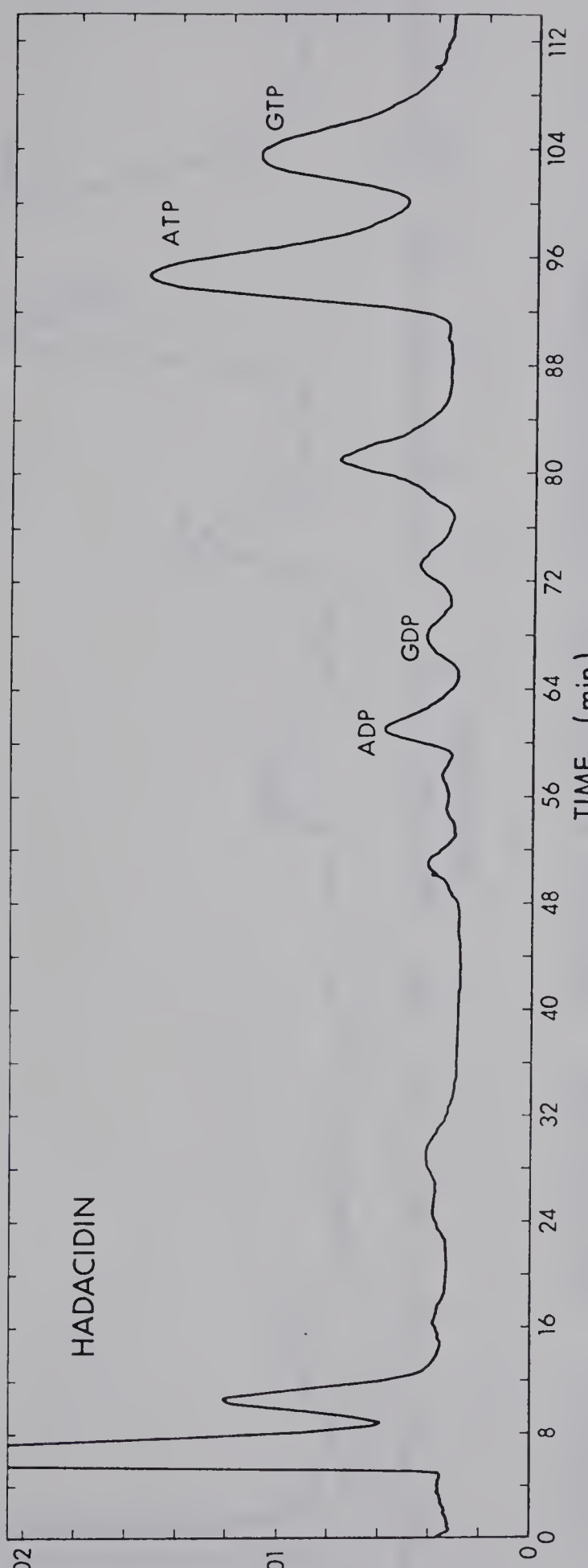

Figure 8. Chromatogram of cells treated with hadacidin.

Mice were injected with 15 µmoles of hadacidin at zero, 1, 2 and 3 hours. Cells were collected at 4 hours.

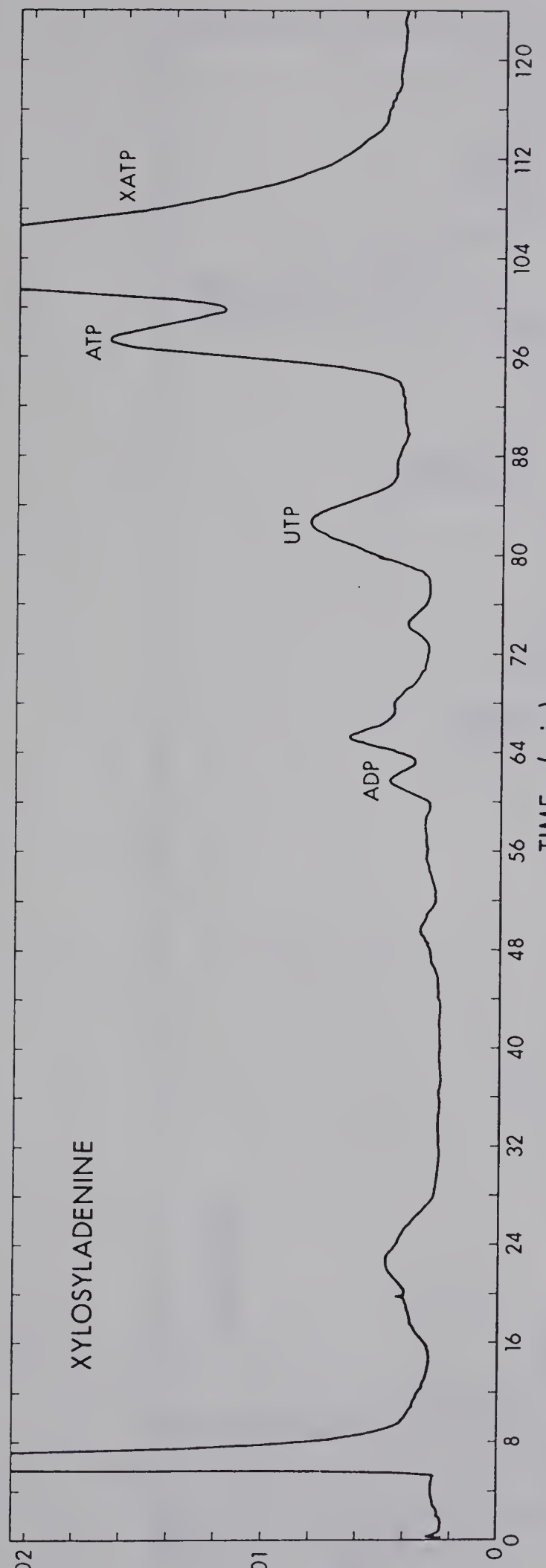

Figure 9. Chromatogram of cells treated with xylosyl adenine.

Mice were injected with 10 µmoles of xylosyl adenine at zero time and with 5 µmoles at 1, 2 and 3 hours. Cells were collected at 4 hours.

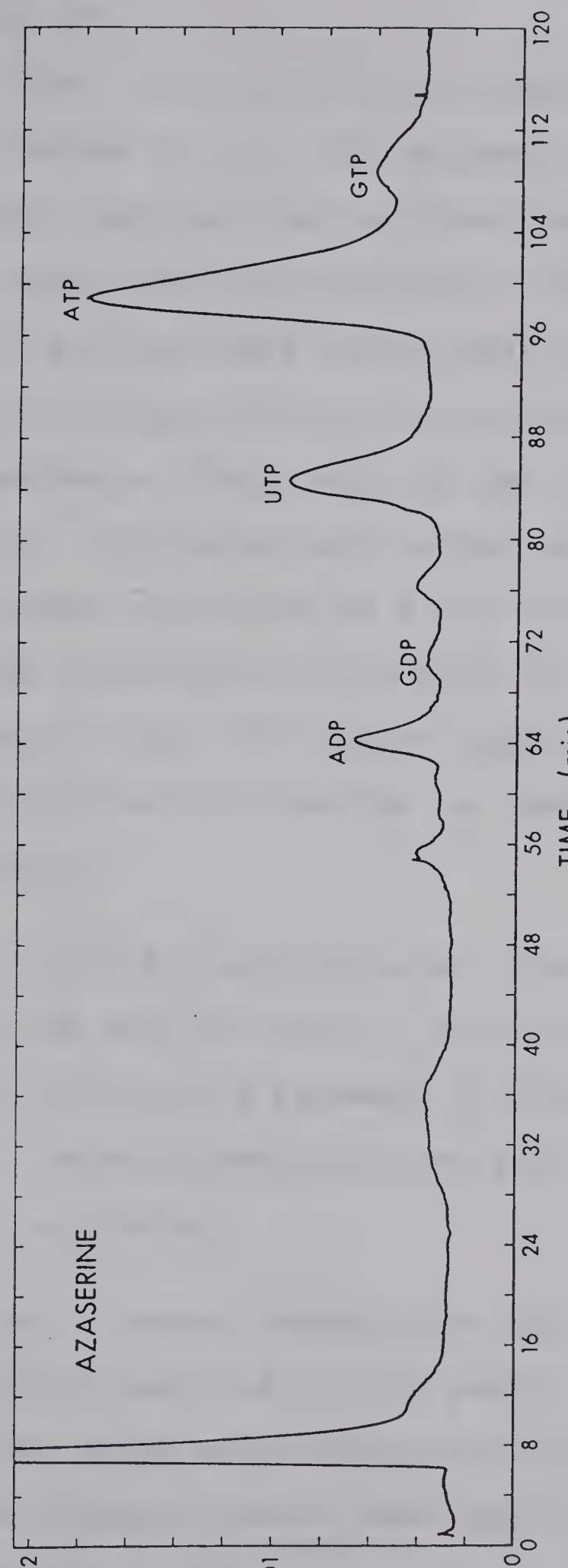

Figure 10. Chromatogram of cells treated with azaserine.

Mice were injected with 20 µg of azaserine and the cells were collected after 4 hours.

xylosyl ATP as ATP.

Fig. 10 shows the nucleotide profile of Ehrlich ascites tumor cells treated *in vivo* with azaserine for 4 hours. Both the ATP and GTP peaks are smaller than those of control cells.

Table 3 gives the concentrations of ATP plus ADP and of GTP in Ehrlich ascites tumor cells after treatment for 4 hours *in vivo* with inhibitors of purine nucleotide synthesis in two separate experiments. Each value is the average of results from three mice. Following each number is the average deviation from the mean, expressed as a per cent of the mean. Because the GDP concentration was very low, it was not measured. Xylosyl ATP and GTP did not separate so the concentration of total acid-soluble guanine is reported instead of that of GTP (Chapter 2).

Calculation of relative activities of alternative pathways

Based on the data of Table 3, the relative activities of the various alternative pathways of purine nucleotide biosynthesis for the maintenance of normal ATP and GTP concentrations were calculated.

Assumptions. Several assumptions had to be made to use the basic approach employed in this study. First it was assumed that the drugs would completely inhibit a particular reaction. The isotope studies described in Chapter 3 show that in fact complete inhibition was not achieved. The nucleotide concentrations measured after treatment with drugs were therefore corrected to account for such incomplete inhibitions.

Table 3. Nucleotide concentrations after drug treatment

Treatment	ATP + ADP (nmoles per gm)		GTP (nmoles per gm)		Total G (nmoles per gm)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None (control)	3386 (3%)*	2998 (9%)	528 (10%)	514 (6%)	512 (6%)	514 (8%)
Mycophenolic acid	2554 (3%)	2732 (3%)	75 (13%)	140 (24%)	-	-
Hadacidin	2191 (2%)	1869 (1%)	971 (6%)	1215 (8%)	-	-
Xylosyladenine	2009 (17%)	1856 (7%)	-	-	682 (3%)	779 (2%)
Azaserine	2117 (6%)	2245 (7%)	333 (5%)	357 (2%)	-	-
Xylo. + Myco.	-+	-	-	-	602 (4%)	566 (11%)

* average deviation from the mean

+ not measured

Another assumption was that the drugs used would be specific, that is other reactions would not be inhibited. In general, the drugs used were specific or their side effects did not affect the calculations. Mycophenolic acid, however, did inhibit GPRT and this was accounted for when calculating the contribution of GPRT to GTP synthesis.

A third assumption was that the inhibition of one pathway would not alter the rate of other pathways. On the basis of isotope studies, the inhibition of purine biosynthesis *de novo* caused an apparent increase in the rate of the phosphoribosyltransferase reactions and inhibition of adenylosuccinate synthetase by hadacidin increased the apparent activity of inosinate dehydrogenase. When necessary these apparent increases were accounted for when calculating the contribution of the alternative pathways.

Lastly it was assumed that nucleotide utilization is via net nucleic acid and coenzyme synthesis and that the drugs would not affect these processes directly or indirectly. On the basis of previous studies, there is no reason to believe that azaserine, hadacidin or mycophenolic acid have direct effects on nucleic acid synthesis. However, xylosyl adenine appeared to cause net degradation of RNA and this was taken into account in the calculations.

Contributions of alternative pathways of GTP synthesis. Table 4 shows the measured or calculated concentrations of GTP when various pathways of synthesis were inhibited. Thus in control cells, when the 4 pathways of GTP synthesis--purine

Table 4. Pathways contributing to GTP

	Control	Azaserine	Mycophenolic Acid	Xylosyl-adenine
Purine biosynthesis *de novo*	+			
HPRT	+	+ (1.7X)*		
GPRT	+	+ (1.9X)*	+	
AMP deaminase	+	+		+
	528	333	0	100‡

* cf. Table 2

‡ Difference between total guanine for xylosyl adenine treatment and xylosyl adenine plus mycophenolic acid treatment.

biosynthesis *de novo*, HPRT, GPRT and AMP deaminase were operating, the GTP concentration was 528 nmoles per gm.

Treatment with azaserine inhibited purine biosynthesis *de novo*, so the pathways contributing to GTP were HPRT at 1.7 times its normal rate (cf. Table 2), GPRT at 1.9 times its normal rate and AMP deaminase. After 4 hours the GTP concentration was reduced to 333 nmoles per gm.

Treatment with mycophenolic acid inhibited the contribution by purine biosynthesis *de novo*, HPRT and AMP deaminase, and reduced the GTP concentration to 75 nmoles per gm. An 80% inhibition of inosinate dehydrogenase by mycophenolic acid reduced the GTP concentration by 453 nmoles per gm; therefore, complete inhibition of inosinate dehydrogenase would have reduced the GTP concentration by 566 nmoles per gm. Since the control value for GTP is 528 nmoles per gm, GPRT contributes little to the maintenance of normal GTP concentration.

Treatment with xylosyl adenine increased the guanine nucleotide concentration. Since xylosyl adenine blocks purine biosynthesis *de novo*, HPRT and GPRT, a reduction in GTP concentration was expected. The possible sources of GTP are from adenine nucleotides via AMP deaminase and from RNA. The contribution of AMP deaminase can be evaluated by comparing the total guanine concentration after xylosyl adenine treatment (682 nmoles per gm) with that after xylosyl adenine plus mycophenolic acid treatment (602 nmoles per gm). The difference, 80 nmoles per gram (corrected to 100 nmoles per gm. for incomplete inhibition by mycophenolic acid), is the contribu-

tion of AMP deaminase.

The contribution of HPRT can be calculated from the GTP concentration after azaserine treatment.

333 nmoles per gm = 1.7 HPRT + 1.9 GPRT + AMP deaminase

333 = 1.7 HPRT + 0 + 100

Solving, HPRT = 137 nmoles per gm. Since the sum of the contribution of all the pathways is 528 nmoles per gm., purine biosynthesis *de novo* contributes 291 nmoles per gm.

The relative importance of each of the synthetic pathways for maintaining normal GTP concentrations calculated from the data just presented was 55% ($\frac{291}{528}$) for purine biosynthesis *de novo*, 26% ($\frac{137}{528}$) for HPRT, 19% ($\frac{100}{528}$) for AMP deaminase and <1% for GPRT. When similar calculations were made for the results of the second experiment the relative contributions were 10% for purine biosynthesis *de novo*, 24% for HPRT, 14% for GPRT and 52% for AMP deaminase.

The reason for the increased calculated contribution of AMP deaminase for the second experiment was that the difference between the total guanine for xylosyl adenine treatment and that for xylosyl adenine plus mycophenolic acid treatment was over twice as much in the second experiment as in the first. This result is discussed below. The calculated contribution of GPRT is greater in the second experiment than in the first because the GTP concentration after mycophenolic acid treatment was nearly twice as much in the second experiment as in the first. The basis

for this difference is not known. However, the lower nucleotide concentration may be more typical because the 86% reduction in GTP concentration caused by mycophenolic acid in the first experiment agreed well with the reduction in GTP of 87% found by Dr. Jeff Lowe (personal communication) for Ehrlich ascites tumor cells treated *in vivo* with mycophenolic acid for 4 hours. Also the average deviation for this GTP concentration was much less in the first experiment than in the second (cf. Table 3).

The calculated contribution for AMP deaminase for the second experiment is unreasonably high. On the basis of *in vivo* isotope studies, approximately 0.6% of adenine nucleotides are converted to guanine nucleotides (C. M. Smith, unpublished results). Assuming a pool size for adenine nucleotides of 3000 nmoles per gm, this is equivalent to 18 nmoles per gm. Using this result for AMP deaminase contribution and the values of the second experiment, the relative contributions are 58% for purine biosynthesis *de novo*, 24% for HPRT, 14% for GPRT and 4% for AMP deaminase.

Table 5 summarizes the percent contributions of each of the pathways of GTP synthesis. The values from the first experiment are reported. APRT and GMP reductase are not directly involved in GTP synthesis.

Table 5. Relative contributions of alternative pathways

Pathway	GTP	ATP
	% Contribution	
Purine biosynthesis *de novo*	55	85
HPRT	26	10
GPRT	<1	
AMP deaminase	19	
APRT		5
GMP reductase		<1
Adenosine kinase		0

Table 6. Pathways contributing to ATP

	Control	Azaserine	Xylosyl adenine		Hadacidin
Purine biosynthesis *de novo*	+				
HPRT	+	+ (1.7X)*			
APRT	+	+ (1.6X)			+
GMP reductase	+	+	+		
Adenosine kinase	+	+	+		+
	3386	2117	1444‡ (minimum)	1949‡ (maximum)	2012

* cf. Table 2

‡ value obtained by subtraction of calculated contribution of nucleotides believed to be derived from RNA.

Contributions of alternative pathways of ATP synthesis. Table 6 shows the measured or calculated concentrations of ATP when alternative synthetic pathways were inhibited. When all of the synthetic pathways--purine biosynthesis *de novo*, HPRT, APRT, GMP reductase and adenosine kinase were operating in untreated control cells, the ATP plus ADP concentration was 3386 nmoles per gm.

Treatment with azaserine inhibited purine biosynthesis *de novo* and reduced the ATP plus ADP concentration to 2117 nmoles per gm after 4 hours.

Treatment with hadacidin inhibited the contribution of purine biosynthesis *de novo*, HPRT and GMP reductase and with only APRT and adenosine kinase operating for 4 hours, the ATP plus ADP concentration was 2191 nmoles per gm. If hadacidin had inhibited adenylosuccinate synthetase completely (cf. Table 2), the ATP plus ADP concentration should have been reduced to 2012 nmoles per gm.

Treatment with xylosyl adenine inhibited purine biosynthesis *de novo*, HPRT and APRT, and reduced the ATP plus ADP concentration to 2009 nmoles per gm after 4 hours. From the results of the effect of xylosyl adenine on GTP concentrations, xylosyl adenine probably causes some RNA degradation.

When purine biosynthesis *de novo*, HPRT and GPRT were blocked by treatment with xylosyl adenine, the total guanine concentration was 682 nmoles per gm. Using the calculated contribution of each of the GTP synthetic pathways and the inhibition by xylosyl adenine of each of these pathways (cf. Table 2), the expected GTP concentration can be calculated as the sum of purine biosynthesis *de novo* (0.02 x 291) + HPRT (0.08 x 137) + AMP deaminase (100) or 117 nmoles per gm. The difference (682 - 117) or 565 nmoles per gm then is the maximum calculated contribution of RNA breakdown to GTP concentration.

A minimum calculated contribution of GTP from RNA degradation, assuming no utilization for RNA or DNA synthesis is the difference between the GTP concentration when all pathways of synthesis are blocked with xylosyl adenine plus mycophenolic acid (588 nmoles per gm) and the control value (528 nmoles per gm); this equals 60 nmoles per gm.

The values just calculated were then used to correct the experimentally determined concentrations of adenine nucleotides. Thus, without the contribution from RNA, the adenine nucleotide concentrations after xylosyl adenine treatment would have a maximum value of 1949 nmoles per gm and a minimum value of 1444 nmoles per gm.

For the purpose of making calculations of the relative contributions of the purine biosynthetic reactions with respect to ATP, adenosine kinase has been assumed to be zero because it could not be specifically inhibited. Thus its contribution is included with that of APRT. In addition, GMP

reductase activity in Ehrlich ascites tumor cells is known to be very low (58) and its contribution is reported as less than 1%. Under these conditions xylosyl adenine, which inhibits purine biosynthesis *de novo*, APRT and HPRT, produces the maximum reduction in ATP concentration.

Using the maximum value for the adenine nucleotide concentration after xylosyladenine treatment (1949 nmoles per gm), calculations were made of the relative importance of the several purine nucleotide synthetic pathways for maintaining normal adenine nucleotide concentration. The difference between the ATP plus ADP concentration after hadacidin treatment (2012) and that after xylosyl adenine treatment is 63 nmoles per gm., and is the contribution of APRT. The difference between ATP plus ADP concentration after azaserine treatment (2117) and that after xylosyl adenine treatment is 168 nmoles per gm., and is equated to 1.6 APRT + 1.7 HPRT. Solving, HPRT = 39 nmoles per gm. Since the total contribution (control - xylosyl adenine) is 1437 nmoles per gm, purine biosynthesis *de novo* contributes 1335 nmoles per gm. As a percent of the total, the relative contributions are 4% ($\frac{63}{1437}$) for APRT, 3% ($\frac{39}{1437}$) for HPRT and 93% ($\frac{1335}{1437}$) for purine biosynthesis *de novo*.

When the minimum value for adenine nucleotide concentration after xylosyl adenine treatment (1444 nmoles per gm) was used, the contributions were 568 nmoles per gm for APRT (29%), 0 for HPRT and 1344 nmoles per gm for purine biosynthesis *de novo* (71%).

Similar calculations were done with the results of the

second experiment. The maximum value of adenine nucleotide concentration gave relative contributions of 5% for APRT, 17% for HPRT and 78% for purine biosynthesis *de novo*. The reason for the difference in the two experiments was that the ATP concentration after azaserine treatment was greater in the second experiment than in the first experiment. The basis for this difference is not known. Also, the average adenine nucleotide value for xylosyl adenine treatment was higher for the first experiment than for the second. This difference was due to one high result in the first experiment, so the value from the second experiment is probably more accurate.

Using the minimum value of adenine nucleotide concentration after xylosyl adenine treatment, the relative contributions were 32% for APRT, 2% for HPRT and 66% for purine biosynthesis *de novo*.

Rates of RNA synthesis. Calculations of the relative contributions of the pathways to the maintenance of normal ATP concentrations were complicated by an apparent degradation of RNA caused by xylosyl adenine. An independent estimate of the normal rate of nucleotide utilization for RNA synthesis (and therefore of nucleotide synthesis) would approximate the expected decrease in nucleotide concentrations if all pathways of nucleotide synthesis were blocked, as for example, by xylosyl adenine. Such an estimation would help to assess the validity of the calculations made above with respect to the effects of xylosyl adenine. Calculation of

the normal rate of nucleotide utilization for RNA synthesis can be made from the RNA content of Ehrlich ascites tumor cells and their generation time at the time of the experiment. Klein *et al.* (59) have measured the increase in Ehrlich ascites tumor cell number *in vivo* after inoculation of different cell numbers. The length of the latency period decreased with increasing inocula and at a dose of 4×10^7 cells there was no detectable latency period. The growth curves were not exponential but the cube root of the cell number was a linear function of time. The following formula for generation time (gt) was derived.

$$gt = \frac{dt}{d(\log N)} \cdot \log 2, \text{where } N = \text{cell number}$$

The inoculum of 4×10^7 cells and the cell number measured after 72 hours in the above study correspond closely to those of this study. Using the published growth curve for an inoculum of 4×10^7 cells and the formula given above for generation time, a generation time of about 47 hours could be calculated for the time the present experiments were done. Using a value of 4.5×10^{-11} gm of RNA per cell (personal communication from Dr. R. von Tigerstrom), the rate of utilization of adenine nucleotides and guanine nucleotides each is 368 nmoles per hour per gm of perchloric acid insoluble material. If all pathways of synthesis were blocked, the guanine nucleotide pool should be depleted in about 1 1/2 hours since the GTP concentration of control cells is 528 nmoles per gm. The ATP concentration would decrease by 1470 nmoles per gm to 1914 nmoles per gm after 4 hours (first experiment). This

ATP value corresponds closely to the calculated "maximum" value of the adenine nucleotide concentration after xylosyl adenine treatment. Thus the relative contributions calculated using the "maximum" value for adenine nucleotide concentration after xylosyl adenine treatment may be more correct than those using the "minimum" value. Averaging the relative contributions for the two experiments, the values are 85% for purine biosynthesis *de novo*, 10% for HPRT and 5% for APRT. These averaged relative contributions are summarized in Table 6. GPRT and AMP deaminase are not directly involved in ATP synthesis.

CHAPTER 5

DISCUSSION

The relative contributions of the alternative pathways of purine nucleotide synthesis calculated in Chapter 4 indicate that although purine biosynthesis *de novo* is the most important pathway for maintaining normal purine nucleotide concentrations, other pathways do make significant contributions. Evidence that purine nucleotides are synthesized by alternative pathways was also provided by the observation that even when purine biosynthesis *de novo* was virtually completely inhibited for one week, growth of mouse tumor cells *in vivo* was inhibited only partially (45). Also, a few cultured cell lines derived from Chinese hamster ovary have been isolated that cannot synthesize purine nucleotides *de novo* (60, 61).

The physiological importance of HGPRT in man is demonstrated by the Lesch-Nyhan syndrome, an X-linked neurological disorder associated with the loss of activity of HGPRT (62, 63). The disease is characterized by overproduction of uric acid, acceleration of purine biosynthesis *de novo*, increased intracellular concentrations of PRPP, developmental retardation, spasticity and compulsive self-mutilation. HGPRT activity in normal human brain and especially in basal ganglia is higher than in other tissues (64) and this may be related to the neurological symptoms of the disease. However, cultured fibroblasts and lymphoblasts from such patients grow at "normal" rates and even in brain, HGPRT deficiency does not retard brain growth or lead to cell death. Even if HGPRT is not essential for the growth of most cells, it may function in nucleotide synthesis from hypoxanthine and guanine produced

by intracellular catabolism of purine nucleotides. Based on a comparison of the absolute and relative amounts of urinary purines excreted by Lesch-Nyhan patients and normal humans, the amount of preformed purines normally utilized by HGPRT was calculated (3). However, these conclusions are seriously complicated by the acceleration of purine biosynthesis *de novo* (and consequent acceleration of hypoxanthine formation) that is known to occur in Lesch-Nyhan patients.

Human cases have been reported who have only 25 to 50% of normal erythrocyte adenine phosphoribosyltransferase activity (65, 66) and as far as is known, this enzyme deficiency has no deleterious consequences.

Cultured cells and animal tumor cells that lack adenosine kinase activity appear to grow at "normal" rates (67, 68, 69). The reutilization (via adenosine kinase) of adenosine produced by intracellular dephosphorylation of AMP has been demonstrated in mouse tumor cells *in vitro* (70, 71), although this was possible only when adenosine deaminase was selectively inhibited. There is considerable evidence that purine derivatives are transported from one tissue to another via the erythrocytes (72, 73), and recent studies have suggested that adenosine may be the compound which is transferred from liver to erythrocytes (73).

Phosphoribosyltransferases may be particularly important in rapidly growing tissues. Both APRT and HGPRT were reported to increase in specific activity following partial hepatectomy in the rat (74). Phosphoribosyltransferase activity was shown

to increase following fertilization of mouse and rabbit ova and increase further in the post-implantation stage of mouse embryo (75).

The calculated relative contributions of alternative pathways reported in Table 6 have varying degrees of accuracy. The percent contribution of purine biosynthesis *de novo* to GTP is likely the most accurate determination. The contributions by HPRT and GPRT are somewhat uncertain since these computations required the measurement of a low GTP concentration. The contribution by AMP deaminase was calculated indirectly as the difference of two large numbers; therefore this value may be somewhat uncertain.

The percent contributions for ATP are less accurate than those for GTP since these calculations required an estimate of the amount of RNA degradation caused by xylosyl adenine. Assuming that the "maximum" values for xylosyl adenine are more accurate than the "minimum" values, the calculated relative contributions are 93% (Exp. 1) and 78% (Exp. 2) for purine biosynthesis *de novo*, 3% (Exp. 1) and 17% (Exp. 2) for HPRT and 4% (Exp. 1) and 5% (Exp. 2) for APRT. Since the ATP plus ADP concentrations after xylosyl adenine treatment in the first experiment varied considerably, the determinations for the second experiment may be more accurate.

An improvement in the experimental method would be to use an inhibitor of PRPP synthetase which does not cause RNA degradation.

As mentioned in chapter 4, one of the assumptions made in using the approach of this study was that the drugs used would not affect the normal utilization of nucleotides for RNA and DNA synthesis. Because the drugs reduced the concentrations of nucleotides, this might result in a reduction of nucleic acid synthesis by limiting the substrate concentrations. If this were the case, the measured nucleotide concentrations would be the result of two factors -- decreased synthesis caused by the drug and decreased utilization for nucleic acid synthesis. The turnover of RNA in Ehrlich ascites tumor cells is considerable (76) and experimental controls should be done to evaluate the effect of drugs on nucleic acid metabolism.

If there was a linear decrease in purine nucleotide concentration for the period of the experiment, the calculated relative contributions of the alternative pathways would be the same at any time point. For azaserine there was a linear decrease in adenine nucleotide concentration. Hadacidin and xylosyl adenine likely cause a linear decrease in purine nucleotide concentrations. However, mycophenolic acid reduces the GTP concentration to a low level within 1 hour (personal communication from Dr. Jeff Lowe). Therefore the relative contribution of the alternative pathways of GTP synthesis should have been determined after a shorter period of inhibition.

The results obtained by this study apply only to Ehrlich ascites tumor cells *in vivo*. However, the same experimental approach could be applied to other biological systems to ascertain the relative contribution of alternative pathways of purine nucleotide synthesis in different cell types.

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